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(57) Abstract

Coacervates of nucleic acids and a polycationic biopolymer are useful for delivery of genes encoding antigens to mammals. The expression of the antigens in the mammal induces an immune response to the antigen. Furthermore, encapsulation of either or both of the antigen protein and a cytokine enhance the immune response. Selection of cytokines can be used to manipulate the type of immune response which is induced.

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GENETIC IMMUNIZATION WITH CO-DELIVERY OF NUCLEIC ACID AND CYTOKINES IN A SINGLE VEHICLE

TECHNICAL FIELD OF THE INVENTION

This invention is related to improved methods of immunization of mammals to achieve cell-mediated and humoral immune responses.

BACKGROUND OF THE INVENTION

Upon immunization, plasmid DNA encoding genes for viral and bacterial antigens can direct the synthesis of the antigens in a native form and effectively present it to the immune system. For instance, potent humoral and cell-mediated immune responses have been induced by HIV-1 DNA vaccination in rodents and non-human primates [1, 2]. The plasmids are typically administered as bolus injections into the muscle or through a gene gun.

Recent findings that DNA immunization can elicit immune responses to viral proteins and confer protective immunity against challenge of the virus in rodent models have stimulated strong interest in optimizing this strategy for developing HIV vaccines [3-5]. Early generations of HIV vaccines that rely on recombinant antigens or killed virus antigens formulated with adjuvants have failed to generate effective CTL responses. DNA immunization offers the advantage of expressing the antigen in its native form that may lead to optimal processing and presentation to antigen presenting cells for induction of both humoral and cellular immune responses.

Although vaccinia vectors are efficient means of delivering the foreign genes *in vivo*, lingering concerns over the safety of these vectors remain. While extensive and elegant research is being conducted to engineer safer viral vectors, non-viral vectors are being increasingly proposed as alternatives. Chief among them for HIV vaccination are direct injection of the plasmid DNA into the muscular or dermal tissues, and bombardment of DNA-coated gold particles through a high pressure gene gun. Unfortunately, transfection efficiency is typically low. Thus there is a need in the art for methods of delivery of DNA which will provide an improved immune response.

The use of cytokines to enhance an immune response to vaccines has attracted intense attention, particularly in the field of cancer immunotherapy. One approach is to introduce cytokine genes directly into the tumor cells [6-23]. The cytokines either enhance the presentation of antigens to T cells or provide additional co-stimulatory signals for T cell activation. In many cases, the locally secreted cytokines elicit an inflammatory reaction that leads to the rejection of the injected tumor cells. In some cases, these genetically-altered tumor cells can generate systemic immunity against subsequent challenge of parental tumor cells, and occasionally even against established micrometastases.

GM-CSF and TNF-α have been found to synergize with IL-12 to enhance induction of cytotoxic T lymphocytes against HIV-1 MN vaccine constructs [1]. In a separate study, codelivery of IL-12 with HIV-1 vaccines in mice resulted in splenomegaly and reduced humoral response, while GM-CSF has the opposite effect [24]. Both cytokines stimulated antigen-specific T cell responses, with a dramatic increase in CTL response observed for the codelivery of IL-12. There is a need in the art for additional methods by which cytokines can be delivered to mammals to stimulate immune responses.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide solid nanospheres for immunization of mammals.

It is another object of the present invention to provide a method for making solid nanospheres for immunization of mammals.

It is an object of the present invention to provide a method for immunizing mammals using solid nanospheres.

These and other objects of the invention are achieved by providing a solid nanosphere of less than 5 μm for genetic immunization of a mammal, comprising a coacervate of a polymeric cation and a polyanion, wherein the polyanion consists of nucleic acids encoding an antigen, wherein the polymeric cation is selected from the group consisting of gelatin and chitosan, and wherein a cytokine is encapsulated in the coacervate.

According to another aspect of the invention a method is provided for immunizing a mammal to raise an immune response to an antigen. The method comprises:

administering to a mammal a solid nanosphere of less than 5 μm comprising a coacervate of a polymeric cation and a polyanion, wherein the polyanion consists of nucleic acids encoding an antigen, wherein the polymeric cation is selected from the group consisting of gelatin and chitosan, and wherein a cytokine is encapsulated in the coacervate.

In another embodiment of the invention a method of forming solid nanospheres for immunization of a mammal is provided. The method comprises the step of:

forming solid nanospheres by coacervation of a polyanion consisting of nucleic acids encoding an antigen and a polymeric cation, wherein the polymeric cation is selected from the group consisting of chitosan and gelatin, wherein said coacervation is done in the presence of a cytokine, whereby the cytokine is encapsulated in said solid nanospheres.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Proliferation of CT-4S cells in response to nanosphere-delivered IL-4. DNA-gelatin nanospheres were synthesized with different loading levels of murine IL-4 (denoted as 0, 100, and 1000U added to the coacervating reaction), were subsequently purified, and incubated with CT-4S cells. 30 hrs. after the addition of nanospheres, the cells were labeled with ^{3}H-thymidine, then radioactivity associated with the cells was measured 18 hrs later. Nanosphere doses are indicated as total DNA. Data points are average of triplicates +/- s.d.

Figure 2. Immunological effects of nanosphere-delivered IL-4. DNA-gelatin nanospheres synthesized with either 0, 1.5, 15 U IL-4 per ug p43-clacZ DNA were injected intramuscularly into groups of 4 mice every two weeks for a total of three immunizations. ELISA assays for anti-β-gal IgG antibody responses were carried out on pooled mice serum at week 4 (after two immunizations). At week 8, lymphocytes from the spleens and lymph nodes from each group were harvested, pooled, and subjected to CTL assays for anti-β-gal responses. a) Anti-β-gal antibody response, b) Anti-β-gal CTL response. The results are average of triplicates +/- s.d.

Figure 3. The anti-β-gal antibody response in mice injected with IL-4 given as nanosphere-encapsulated vs. free form. Groups of 4 mice were injected i.m. with nanospheres mixed with 20 U of free IL-4,. nanospheres encapsulated with 20 U IL-4 per 1 mg p43-clacZ DNA, DNA, or DNA mixed with 20 U of free IL-4. Each group was vaccinated twice at four weeks intervals with 1 mg total DNA. At week 8, the sera from individual groups were pooled then assayed for total IgG anti-β-gal antibody. Data are average of triplicates +/- s.d.

Figure 4. Potentiation of CTL responses using nanosphere-encapsulated IL-2 and γ-INF. Groups of 4 mice were injected once i.m. with 2 mg of total p43-clacZ DNA either as nanospheres, nanospheres encapsulated with IL-2 (20 U per 1 mg p43-clacZ DNA), nanospheres encapsulated with IL-2 and γ-INF

(100 U per 1 mg pg 43-clacZ DNA), or 'naked' DNA. Standard CTL assays were carried out at week 4. Data are average +/- s.d.

Figure 5. Survival of mice infected with Ebola virus following vaccination with Ebola NP pDNA or Ebola GP pDNA delivered by nanosphere. 8-wk old BALB/c mice (10 mice per group) were immunized i.m. in the tibialis anterior with three monthly injections of nanospheres containing 0.5 μg (-□-) or 3 μg (-■-) Ebola NP pDNA; 0.5μg (-○-) or 3μg (-●-) Ebola GP pDNA; or 3 μg control WRG7077 pDNA (-△-) (vector without the Ebo NP or GP gene insert). The mouse-adapted Ebola virus challenge system and Ebola pDNA vector contructs are described in detail in Vanderzanden, 1998, *Virology 246:*134-144. Survival rates were tabulated after challenge with 30 x LD_{50} of live mouse-adapted Ebola Zaire virus at week 12. No deaths were observed beyond day 10.

DETAILED DESCRIPTION

The disclosure of prior U.S. patent applications Serial Nos. 08/265,966, and 08/657,913 are expressly incorporated herein.

To improve the delivery of nucleic acids for immunizing animals, we have developed a non-viral gene delivery system (nanospheres) synthesized by the salt-induced complex coacervation of nucleic acids with either gelatin or chitosan. Cytokines are co-encapsulated in the nanospheres to enhance the efficacy of genetic vaccination and to produce a desired immune response. The ultimate immune response elicited by the vaccine is influenced by the interaction between the specific and non-specific immune mechanisms. The type of immune cells attracted to the site of vaccination, for example, can determine the eventual antitumor immunity induced by a tumor-associated antigen. The repertoire of these cells is in turn governed by the temporal and spatial distribution of cytokines. By co-encapsulating the cytokine genes in the nanospheres, the temporal and spatial distribution of the cytokines can be further altered. This can direct the immune response toward a specific immune arm, such as the Th1 or Th2 pathway. This strategy can be used, for example,

to modulate the immune response against HIV infection by emphasizing the humoral or cellular arm.

Our approach has elements in common with the cationic lipid-DNA condensates and ligand-polycation-DNA complexes in the physical delivery of DNA to cells through endocytosis, but differs in the important aspect that the nucleic acids are also physically entrapped in a solid matrix and the coacervate is stable extracellularly. This gene delivery system has several attractive features: 1) ligands can be conjugated to the nanosphere to stimulate receptor-mediated endocytosis and potentially to target cell/tissue; 2) lysosomolytic agents can be incorporated to promote escape of intact DNA into cytoplasm; 3) other bioactive agents such as RNA, oligonucleotides, proteins, or multiple plasmids can be co-encapsulated. Relevant to vaccine application is that the protein antigen may also be co-encapsulated in the nanosphere for potential augmentation of immune response through class II presentation, and as suggested by recent findings, as well as class I presentation when the nanosphere is internalized by antigen presenting cells; 4) bioavailability of the nucleic acids can be improved because of protection from serum nuclease degradation by the matrix, and there is little release of the nucleic acids until the nanosphere is sequestered into the endolysosomal pathway. There is also the potential of intracellular sustained release of nucleic acids that may provide more prolonged expression of the gene product; 5) the nanosphere is stable in plasma electrolytes, and can be lyophilized without loss of bioactivity. Hence the nanospheres can be handled more like conventional pharmaceutical formulations in terms of production, reproducibility, and storage.

We have demonstrated that the role of paracrine delivery of cytokines by gene transduction can be fulfilled by biodegradable controlled release nanospheres. This approach is less labor intensive, because the necessity of individualized gene transfer is eliminated. It is more controllable, because consistent levels of cytokines can be achieved more readily than with gene transfer, and the rate and duration of the cytokine delivery can be varied by

changing the properties of the nanospheres. It is also more versatile, because multiple cytokines can be readily used, and the tumor antigen to cytokine ratio can be varied easily. These beneifts also apply to other types of vaccinations such as HIV vaccination.

Complex coacervation is a process of spontaneous phase separation that occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. The electrostatic interaction between the two species of macromolecules results in the separation of a coacervate (polymer-rich phase) from the supernatant (polymer-poor phase). This phenomenon can be used to form nanospheres and encapsulate a variety of compounds. The encapsulation process can be performed entirely in aqueous solution and at low temperatures, and has a good chance, therefore, of preserving the bioactivity of the encapsulant.

According to the present invention, gelatin or other polymeric cation having a similar charge density to gelatin, is used to complex with nucleic acids to form nanospheres. This includes, but is not limited to gelatin and chitosan. The source of gelatin is not thought to be critical; it can be from bovine, porcine, human, or other animal source. Typically the polymeric cation has a molecular weight of between 19,000-30,000. Poly-L-lysine or chitosan may be particularly useful as the polymeric cation of the present invention. Polyamino acids, synthetic or naturally occurring, can also be used, such as polylysine, poly-lysine-poly-arginine, polyarginine, protamine, spermine, spermidine, etc. Polysaccharides may also be used. Desirably sodium sulfate is used to induce the coacervation of polymeric cation and nucleic acids. Ethanol can also be used at a concentration of about 40 to 60% to induce coacervation. Chondroitin sulfate can also be incorporated into the nanosphere, which is especially beneficial if one desires other substances such as drugs and lysosomolytic agents to be incorporated in the nanosphere. Typically the concentration of chondroitin sulfate is between about 0.005% and 0.1%.

It is preferred that the nanospheres be less than 5 microns. More preferred are nanospheres of less than 3 microns, and even more preffered are nanospheres which are less than 2, 1, 0.5, and 0.1 microns. While size can be effected by the conditions of coacervation and the size of the component polyanion and polycation, nanospheres of the desired size can also be size selected using a technique which separates the nanospheres on the basis of size.

Targeting ligands, if desired, can be directly bound to the surface of the nanosphere or can be indirectly attached using a "bridge" or "spacer". Because of the amino groups provided by the lysine groups of the gelatin, the surface of the nanospheres can be easily derivatized for the direct coupling of targeting moieties. For example, carbo-diimides can be used as a derivatizing agent. Alternatively, spacers (linking molecules and derivatizing moieties on targeting ligands) such as avidin-biotin can be used to indirectly couple targeting ligands to the nanospheres. Biotinylated antibodies and/or other biotinylated ligands can be coupled to the avidin-coated nanosphere surface efficiently because of the high affinity of biotin ($k_a \sim 10^{15}$ M^{-1}) for avidin (Hazuda, et al., 1990, Processing of precursor interleukin 1 beta and inflammatory disease, *J. Biol. Chem.*, **265**:6318-22; Wilchek, et al., 1990, Introduction to avidin-biotin technology, *Methods In Enzymology*, **184**:5-13). Orientation-selective attachment of IgGs can be achieved by biotinylating the antibody at the oligosaccharide groups found on the F_C portion (O'Shannessy, et al., 1984, A novel procedure for labeling immunoglobulins by conjugation to oligosaccharides moieties, *Immunol. Lett.*, **8**:273-277). This design helps to preserve the total number of available binding sites and renders the attached antibodies less immunogenic to F_C receptor-bearing cells such as macrophages. Spacers other than the avidin-biotin bridge can also be used, as are known in the art. For example, Staphylococcal protein A can be coated on the nanospheres for binding the F_C portions of immunoglobulin molecules to the nanospheres.

Cross-linking of linking molecules or targeting ligands to the nanosphere is used to promote the stability of the nanosphere as well as to

covalently affix the linking molecule or targeting ligand to the nanosphere. The degree of cross-linking directly affects the rate of nucleic acids released from the nanospheres. Cross-linking can be accomplished using glutaraldehyde, carbodiimides such as EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, DCC (N,N'-dicyclohexylcarbodiimide), carboxyls (peptide bond) linkage, bis (sulfosuccinimidyl) suberate, dimethylsuberimidate, etc.

Targeting ligands according to the present invention are any molecules which bind to specific types of cells in the body. These may be any type of molecule for which a cellular receptor exists. Preferably the cellular receptors are expressed on specific cell types only. Examples of targeting ligands which may be used are hormones, antibodies, cell-adhesion molecules, saccharides, drugs, and neurotransmitters.

The nanospheres of the present invention have good loading properties. Typically, following the method of the present invention, nanospheres having at least 5% (w/w) nucleic acids can be achieved. Preferably the loading is greater than 10 or 15% nucleic acids. Often nanospheres of greater than 20 or 30%, but less than 40 or 50% nucleic acids can be achieved. Typically loading efficiencies of nucleic acids into nanospheres of greater than 95% can be achieved.

The method of the present invention involves the coacervation of polymeric cations and nucleic acids. Because this process depends on the interaction of the positively charged polymeric cations and the negatively charged nucleic acids it can be considered as a complex coacervation process. However, sodium sulfate (or ethanol) induces the coacervation reaction by inducing a phase transition, and therefore it could also be considered as a simple coacervation reaction. Nucleic acids are present in the coacervation mixture at a concentration of between 1 ng/ml to 500 μg/ml. Desirably the nucleic acids are at least about 2-3 kb in length. Sodium sulfate is present at between 7 and 43 mM. Gelatin or other polymeric cation is present at between about 2 and 7% in the coacervation mixture.

An attractive nanosphere delivery system requires a delicate balance among factors such as the simplicity of preparation, cost effectiveness, nucleic acids loading level, controlled release ability, storage stability, and immunogenicity of the components. The gene delivery system described here may offer advantages compared to other particulate delivery systems, including the liposomal system. The problems of instability, low loading level, and controlled release ability are better resolved with the polymeric nanosphere systems. Gelatin has received increasing biologic use ranging from surgical tissue adhesive (Weinschelbaum, et al., 1992, Surgical treatment of acute type A dissecting aneurysm with preservation of the native aortic valve and use of biologic glue. Follow-up to 6 years, *J. Thorac. Cardiovasc. Surg.*, **130**:369-74) to quantitative immunohistochemical assays (Izumi, et al., 1990, Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field, *J. Clinical Microbiol.*, **28**:525-9) and as drug delivery vehicle (Tabata, et al., 1991, Effects of recombinant alpha-interferon-gelatin conjugate on in vivo murine tumor cell growth, *Cancer Res.*, **51**:5532-8), due to its biocompatibility and enzymatic degradability *in vivo*. Compared to other synthetic polymeric systems, such as the extensively studied polylactic/polyglycolic copolymers, the mild conditions of nanosphere formulation are appealing. Unlike the solvent evaporation and hot-melt techniques used to formulate synthetic polymeric nanospheres, complex coacervation requires neither contact with organic solvents nor heat. It is also particularly suitable for encapsulating bio-macromolecules such as nucleic acids not only through passive solvent capturing but also by direct charge-charge interactions.

Unlike viral vectors, which cannot deliver genes larger than 10 kb, the nanosphere delivery system of the present invention does not have such size limitations. Nucleic acid molecules of greater than about 2 kb can be used, and nucleic acid molecules even greater than 10 kb may be used. Typically the nucleic acid will be greater than 300 bases, and typically geater than 0.5, 1, 2, 5, or 10 kb. Typically the nucleic acid molecule will be less than 200, 100, or 50 kb.

In general, the range of possible targets is dependent on the route of injection, *e.g.*, intravenous or intraarterial, subcutaneous, intra-peritoneal, intrathecal, etc. For systemic injections, the specificity of this delivery system is affected by the accessibility of the target to blood borne nanospheres, which in turn, is affected by the size range of the particles. Size of the particles is affected by temperature, component concentration, and pH in the coacervation mixture. The particles can also be size-fractionated, *e.g.*, by sucrose gradient ultracentrifugation. Particles with size less than 150 nanometers can access the interstitial space by traversing through the fenestrations that line most blood vessels walls. Under such circumstances, the range of cells that can be targeted is extensive. An abbreviated list of cells that can be targeted includes the parenchymal cells of the liver sinusoids, the fibroblasts of the connective tissues, the cells in the Islets of Langerhans in the pancreas, the cardiac myocytes, the Chief and parietal cells of the intestine, osteocytes and chondrocytes in the bone, keratinocytes, nerve cells of the peripheral nervous system, epithelial cells of the kidney and lung, Sertoli cells of the testis, etc. The targets for particles with sizes greater than 0.2 microns will be confined largely to the vascular compartment. Here, the targetable cell types include erythrocytes, leukocytes (i.e. monocytes, macrophages, B and T lymphocytes, neutrophils, natural killer cells, progenitor cells, mast cells, eosinophils), platelets, and endothelial cells.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Immunological responses of pCI-clacZ plasmid DNA vectors. Anti β-gal immune responses elicited by two lacZ expression vectors differing primarily by the presence of the adeno-associated virus inverted terminal repeats (AAV-ITR) were evaluated. The insertion of the CMV-intronA-lacZ (CMV/lacZ)

cassette of p43-clacZ into an expression vector lacking the AAV-ITR (pCI-neo) resulted in markedly diminished anti-β-gal antibody and CTL responded in mice immunized i.m. with 'naked' DNA. At high DNA dose (100 mg). the p43-clacZ vector still out-performed the pCI-clacZ vector, although the difference in responses were significantly smaller as compared to that observed at the low dose (1 mg). With the exception of the neomycin resistance gene ('*neo*') on pCI-clacZ, the only difference between pCI-clacZ and p43-clacZ is the AAV-ITR flanking the CMV/lacZ cassette. Since the *neo* gene does not contribute to lacZ gene expression, and it has no known immunological consequences in studies where it has been employed, these results suggest that the observed differences in anti-β-gal immunological responses may be attributable to the presence of the AAV-ITR. *In vitro* transfection studies using Lipofectamine reagents carried out on 293 cells showed no differences in the gene expression levels between these two vectors (data not shown), suggesting that the differences in immune response was not related to the level of gene expression.

EXAMPLE 2

Immunological responses of pCI-clacZ plasmid DNA vectors. Anti β-gal immune responses elicited by two lacZ expression vectors differing primarily by the presence of the adeno-associated virus inverted terminal repeats (AAV-ITR) were evaluated. The insertion of the CMV-intronA-lacZ (CMV/lacZ) cassette of p43-clacZ into an expression vector lacking the AAV-ITR (pCI-neo) resulted in markedly diminished anti-β-gal antibody and CTL responded in mice immunized i.m. with 'naked' DNA . At high DNA dose (100 mg). the p43-clacZ vector still out-performed the pCI-clacZ vector, although the difference in responses were significantly smaller as compared to that observed at the low dose (1 mg). With the exception of the neomycin resistance gene ('*neo*') on pCI-clacZ, the only difference between pCI-clacZ and p43-clacZ is the AAV-ITR flanking the CMV/lacZ cassette. Since the *neo* gene does not contribute to lacZ gene expression, and it has no known immunological

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EXAMPLE 3

Immunological effects of co-delivered IL-4. Co-delivery of IL-4 was evaluated in mice vaccinated with p43-clacZ DNA nanospheres containing different amounts of IL-4. Nanospheres synthesized with either 0, 1.5, 15 U IL-4 per mg DNA were injected i.m. into groups of 4 mice every two weeks for a total of three immunizations. ELISA assays on mouse serum carried out at week 4 showed a progressively greater anti-β-gal antibody response in groups receiving nanospheres with increasing IL-4 loading levels (Fig. 2). At week 8, CTL assays carried out using lymphocytes from these mice showed an IL-4 dose-related reduction anti-β-gal CTL responses. Remarkably, complete shut-down of anti-β-gal CTL response was observed at the 15 U IL-4 dose. It should be noted the IL-4 dose at which immunological effects were observed, *i.e.* ~ 1-15 U per vaccination represented two to three orders of magnitude lower than what has been employed in other studies to yield an IL-4 immunological effect. This results suggest that IL-4 can be effective at lower doses when microencapsulated in the DNA-gelatin nanospheres.

EXAMPLE 4

Cytokine production lymphocytes from nanosphere-vaccinated mice. To further characterize the immunological response of mice vaccinated with nanospheres containing different doses of IL-4, the lymphocytes were harvested, stimulated with β-gal protein, then analyzed for IL-4 and γ-INF

production. Standard ELISA assays carried out on 3-day cultured serum revealed that mice immunized with nanospheres with increasing IL-4 content produced proportionately more IL-4 upon re-stimulation with antigens. In these serum. a dose-related decrease in γ-INF production was observed. Because the production of IL-4 is associated with actively proliferating T_h2 helper cells, while γ-INF production is associated with T_h1 helper cells, it is concluded that in mice vaccinated with nanosphere IL-4, the T_h2 helper cells response was potentiated while T_h1 helper cell response was depressed. This result, in combination with a dose-related potentiation of anti-β-gal antibody and a concomitant inhibition of CTL responses, is consistent with an IL-4 mediated immunological switch form a T_h1-weighed response to that of a T_h2.

EXAMPLE 5

The effects of IL-4 given as nanosphere-encapsulated vs. free form. The effectiveness of IL-4 in potentiating an antibody response was examined in mice vaccinated with nanospheres containing IL-4 either as microencapsulated or free form. ELISA assays carried out on mouse serum at week 8 (after two immunizations) showed that those injected with nanospheres mixed with free IL-4 failed to elicit an enhancement in anti-β-gal antibody response (Fig. 3). In contrast, mice vaccinated with nanosphere-encapsulated IL-4 generated a marked enhancement of antibody response. Moreover, mice injected with 'naked' DNA in the presence of the same IL-4 dose also failed to generate an immune enhancement as compared to those vaccinated with DNA alone. The data suggest that the IL-4 dose employed in the above experiment was effective in potentiating an antibody response only as nanosphere-encapsulated form.

EXAMPLE 6

Potentiation of the CTL response using nanosphere-encapsulated IL-2 and γ-INF. The feasibility of immune response modulation using nanosphere co-delivered cytokines was further demonstrated by examining the effects of

nanosphere-encapsulated IL-2 and γ-INF on the CTL responses. Mice immunized with a single injection of nanospheres containing p43-clacZ DNA alone (2 mg total DNA), with IL-2, or with IL-2 and γ-INF were examined for the generation of anti-β-gal CTL responses at week 4. Mice vaccinated with nanosphere alone or naked DNA generated poor CTL response (Fig. 4). This was consistent with our previous studies which showed at least two or three immunizations were necessary for strong CTL responses. However, when IL-2 was included in the nanospheres, an enhancement in CTL response was observed. The CTL response was potentiated synergistically when γ-INF was co-delivered with IL-2 in the nanospheres. The inclusion of both IL-2 and γ-INF in nanosphere improved the anti-β-gal CTL response from 25% lysis (at an E:T ratio of 64:1) to at least 65% with just a single immunization.

EXAMPLE 7

Protection against a lethal challenge dose of live Ebola virus in nanosphere-vaccinated mice. Ebola virus, a negative sense RNA virus, causes severe hemorrhagic fever leading to high mortality rates in infected humans. We tested the efficiency of nanosphere vaccination with DNA encoding the Ebola virus ribonucleocapsid NP protein (Ebo NP) and the envelope GP glycoprotein (Ebo GP) antigens. Previous DNA immunization studies have suggested that the protective efficacy of the Ebo NP antigen is correlated with a CTL repsonse, whereas protection assoociated with the Ebo GP antigen is associated with both strong antibody and CTL repsonses. Mice vaccinated with 0.5 or 3 μg of nanosphere DNA encoding Ebola NP or Ebola GP were challenged with 30 x LD_{50} of mouse-adapted live Ebola Zaire strain. The survival rate was better with each antigen than with the vector control and was significantly greater with the higher dose ($p<0.01$) (Fig. 5). A higher degree of protection was achieved with Ebola NP vaccination than with Ebola GP (90% vs 40%). The geometric means anti-GP or anti-NP antibody titers of immunized mice were low, 1 +/- 0.1 x 10^2. Vaccination with DNA nanospheres was at least as efficient as the gene gun vaccination method. A parallel challenge experiment using the NP antigen given as *PowerJect-Xr*™

gene gun DNA (3 μg dose, three total vaccinations), showed a protection level of 80%.

We have demonostrated the effectiveness of nanosphere-based genetic vaccine delivery in a mouse-adapted Ebola virus model system. The protection observed against challenge with 30 times the LD_{50} of live Ebola virus was greater following immunization with a nucleoprotein antigen than with an envelope glycoprotein antigen. This observation may reflect the expected greater presentation of a nucleoprotein peptide as a cytotoxic epitope than that of an envelope protein. A similar level of protection has been obtained by gene gun immunization with the same amount of Ebola DNA, and it remains to be determined which of these procedures can be developed into an effective vaccine with respect to the important features of a vaccine system, including formulation, ease of administration, safety, co-delivery of immune modulators, and protection of DNA. The results of the present study suggest, however, that the nanosphere may provide an important new type of DNA vaccine delivery system that is of particular value in disease states in which a specific immune response phenotype is required.

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CLAIMS

1. A solid nanosphere of less than 5 μm for genetic immunization of a mammal, comprising a coacervate of a polymeric cation and a polyanion, wherein the polyanion consists of nucleic acids, wherein at least a portion of the nucleic acids encode an antigen, and wherein a cytokine is encapsulated in the coacervate.

2. The nanosphere of claim 1 wherein at the polymeric cation is gelatin.

3. The nanosphere of claim 1 wherein at the polymeric cation is chitosan.

4. The nanosphere of claim 1 wherein a cell targeting ligand is attached to said nanosphere.

5. The nanosphere of claim 2 wherein the targeting ligand is covalently attached to said nanosphere by means of glutaraldehyde cross-linking.

6. The nanosphere of claim 1 wherein at least a portion of the nucleic acids encode a cytokine.

7. The nanosphere of claim 1 wherein said polymeric cation is chitosan.

8. The nanosphere of claim 1 wherein the cytokine is selected from the group consisting of GM-CSF, TNF-α, IL-12, IL-4, γ-IFN, and combinations thereof.

9. The nanosphere of claim 1 wherein the antigen is a viral antigen.

10. The nanosphere of claim 1 wherein the antigen is a bacterial antigen.

11. The nanosphere of claim 1 wherein the antigen is a tumor-associated antigen.

12. The nanosphere of claim 1 wherein the antigen is also encapsulated in the coacervate.

13. A method of immunizing a mammal to raise an immune response to an antigen, comprising:

administering to a mammal a solid nanosphere of less than 5 μm comprising a coacervate of a polymeric cation and a polyanion, wherein the polyanion consists of nucleic acids encoding an antigen, and wherein a cytokine is encapsulated in the coacervate.

14. The method of claim 13 wherein the polymeric cation is selected from the group consisting of gelatin and chitosan,

15. The method of claim 13 wherein the administering is performed by injection into a muscle.

16. The method of claim 13 wherein the administering is performed by subcutaneous injection.

17. The method of claim 13 wherein the administering is performed by bombardment with the nanospheres from a high pressure gene gun.

18. A method of forming solid nanospheres for immunization of a mammal, comprising the steps of:

forming solid nanospheres by coacervation of a polyanion consisting of nucleic acids encoding an antigen and a polymeric cation, wherein the polymeric cation is selected from the group consisting of chitosan and gelatin, wherein said coacervation is done in the presence of a cytokine, whereby the cytokine is encapsulated in said solid nanospheres.

19. The method of claim 18 further comprising the steps:

cross-linking a cell targeting ligand to the nanospheres.

20. The method of claim 18 wherein the coacervation is performed in the presence of sodium sulfate.

21. The method of claim 18 wherein the polymeric cation is gelatin.

22. The method of claim 18 wherein the polymeric cation is chitosan.

23. The method of claim 18 wherein a targeting ligand is adhered to the surface of said nanosphere, said targeting ligand being selected from the group consisting of antibodies, hormones, cell-adhesion molecules, saccharides, drugs, and neurotransmitters.

24. The method of claim 18 wherein the gelatin is present at a concentration of about 2-7% in the step of coacervation.

25. The method of claim 18 wherein the nucleic acids are present in a concentration of 1 ng/ml to 500 μg/ml in the step of coacervation.

26. The method of claim 18 wherein the concentration of sodium sulfate is between about 7 and 43 mM in the step of coacervation.

27. The nanosphere of claim 1 wherein said nucleic acids encode a gene of 2 to 10 kb.

FIG. 1

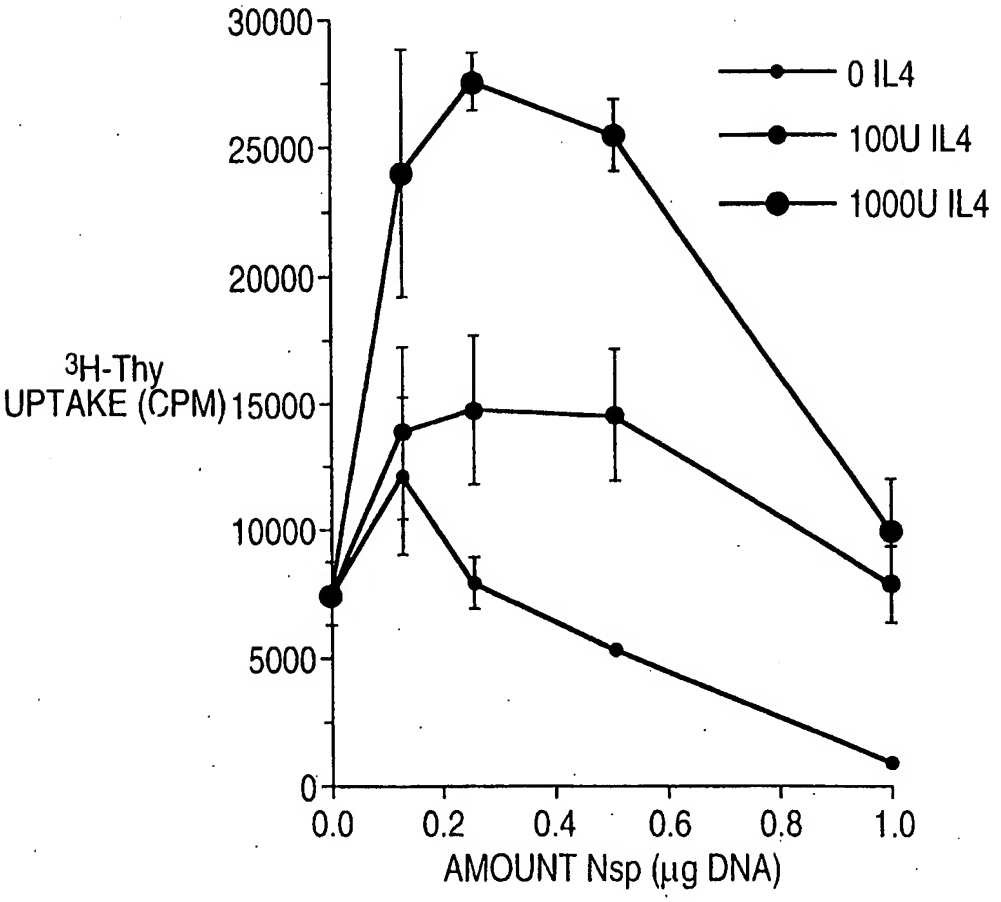

30000
25000
20000
15000
10000
5000
0
^{3}H-Thy
UPTAKE (CPM)
0.0
0.2
0.4
0.6
0.8
1.0
AMOUNT Nsp (µg DNA)
0 IL4
100U IL4
1000U IL4

FIG. 2A

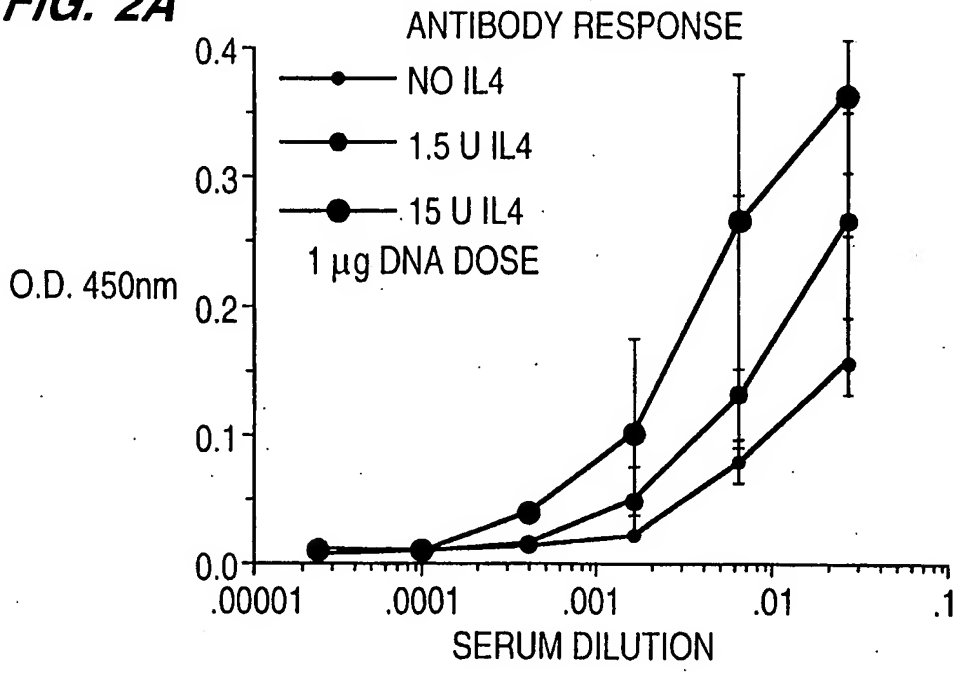
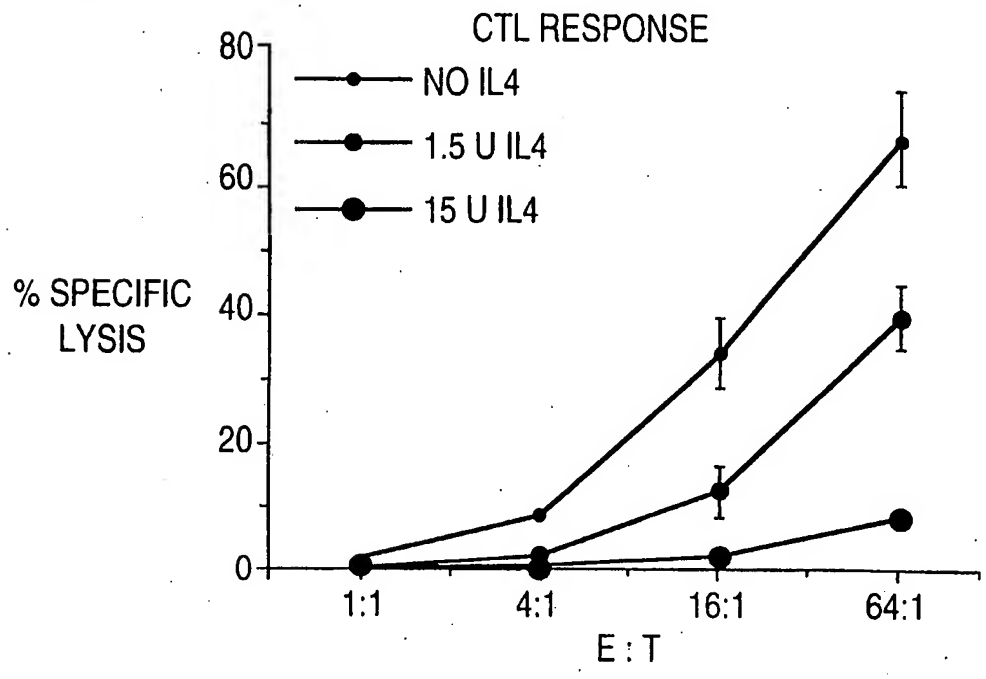

ANTIBODY RESPONSE
NO IL4
1.5 U IL4
15 U IL4
1 µg DNA DOSE
O.D. 450nm
0.4
0.3
0.2
0.1
0.0
.00001
.0001
.001
.01
.1
SERUM DILUTION

FIG. 2B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NO IL4
1.5 U IL4
15 U IL4
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80
60
40
20
0
1:1
4:1
16:1
64:1
E : T

FIG. 3

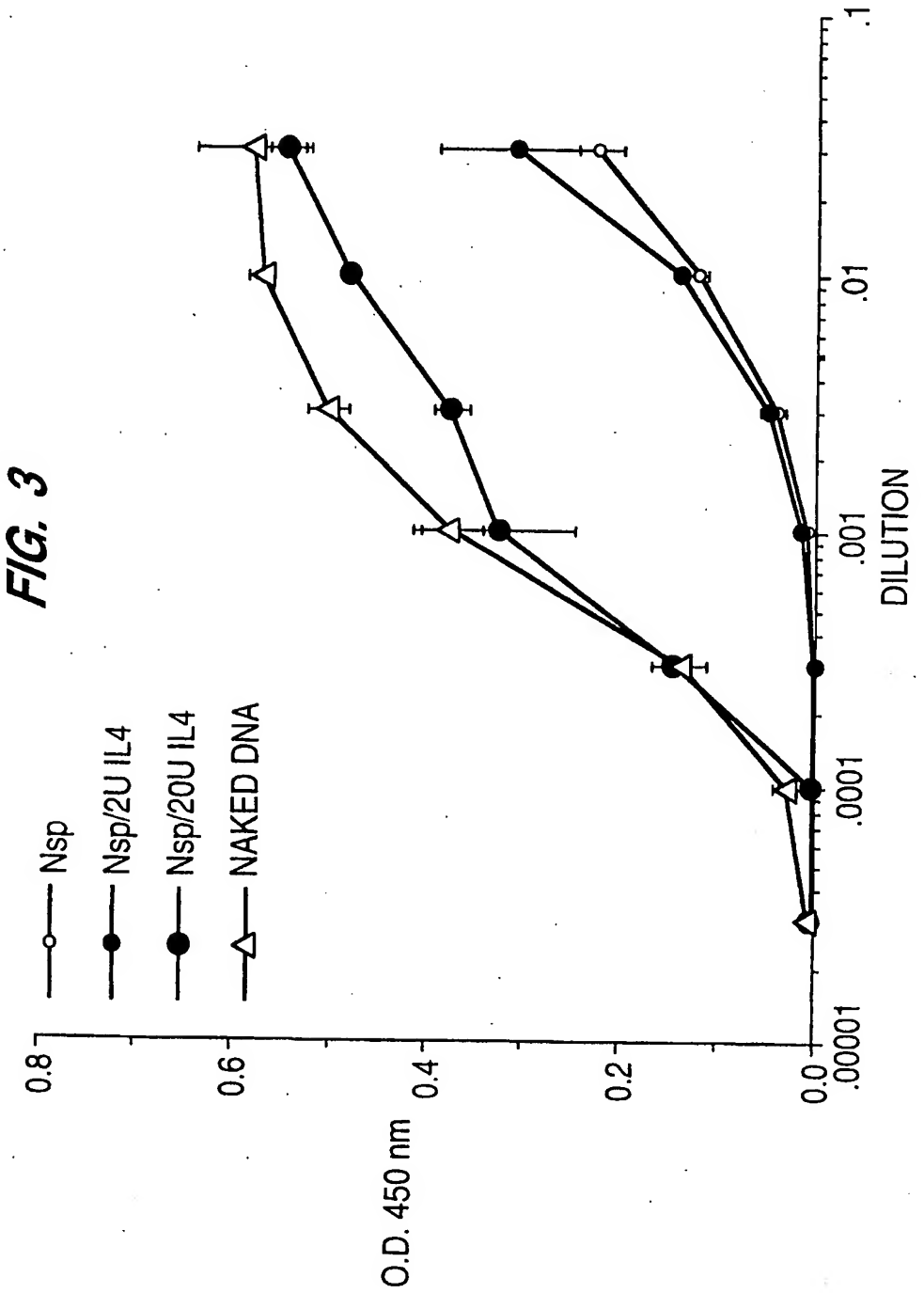

Nsp
Nsp/2U IL4
Nsp/20U IL4
NAKED DNA
0.8
0.6
0.4
0.2
0.0
O.D. 450 nm
.00001
.0001
.001
.01
.1
DILUTION

FIG. 4

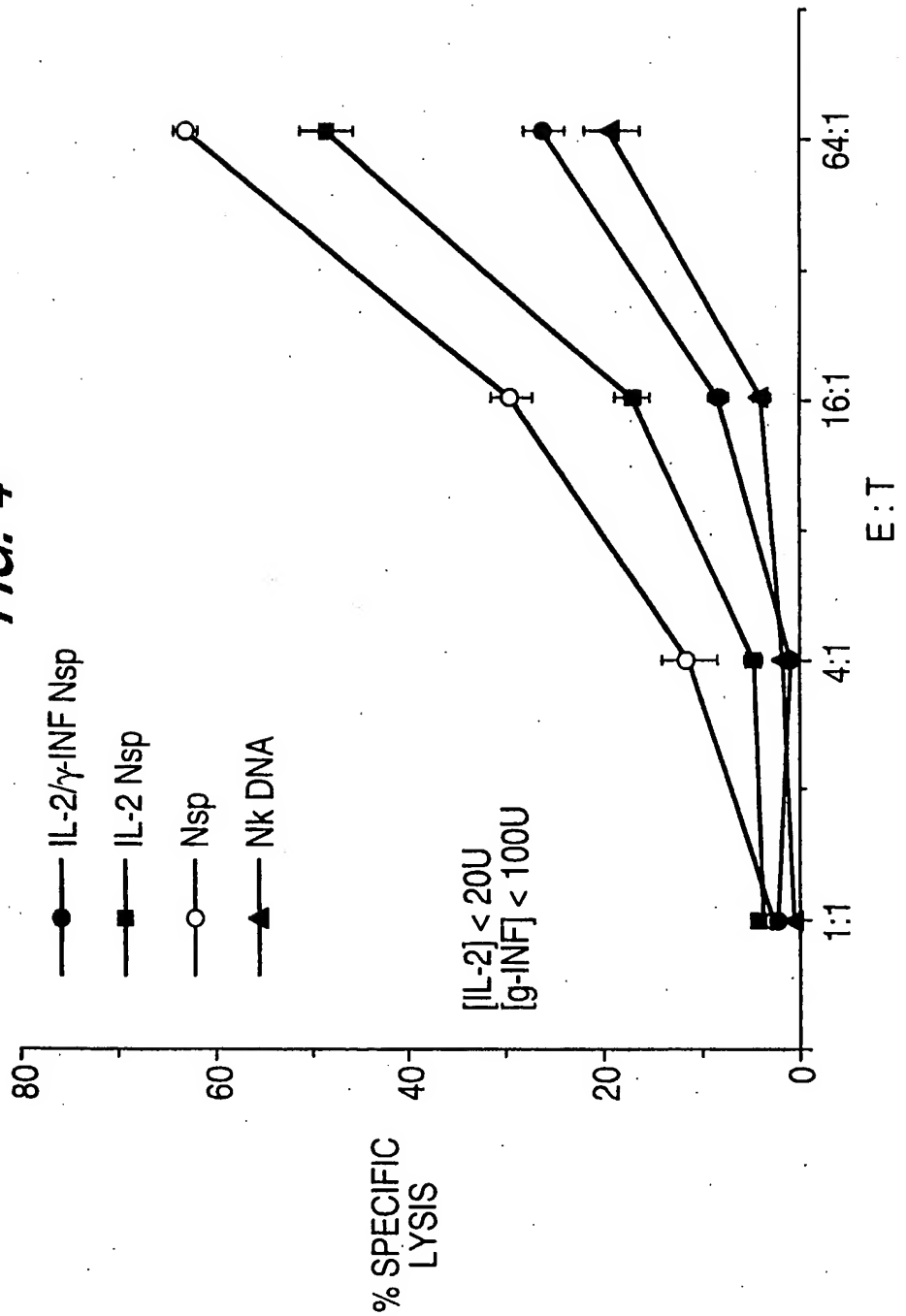

IL-2/γ-INF Nsp
IL-2 Nsp
Nsp
Nk DNA
[IL-2] < 20U
[g-INF] < 100U
% SPECIFIC LYSIS
0
20
40
60
80
1:1
4:1
16:1
64:1
E : T

FIG. 5A

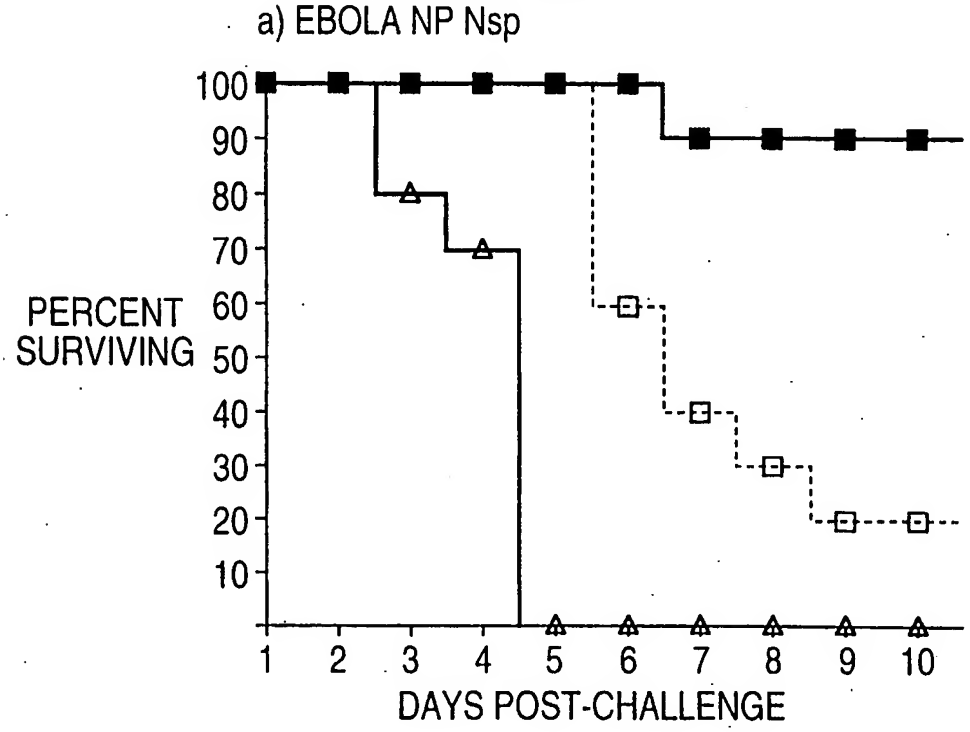
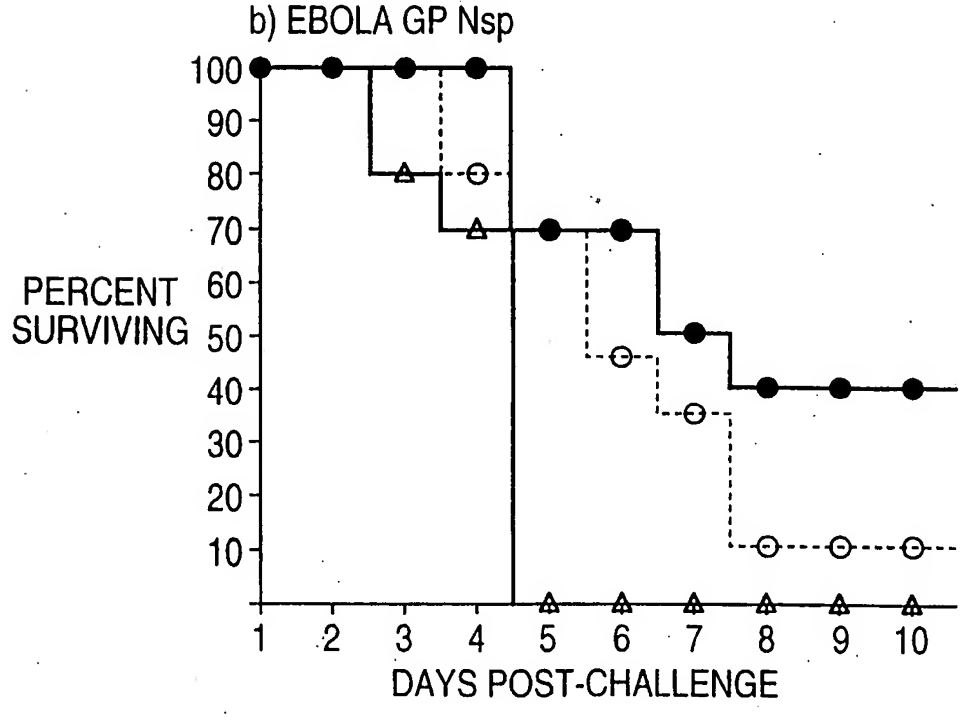

a) EBOLA NP Nsp
PERCENT SURVIVING
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10
DAYS POST-CHALLENGE

FIG. 5B


b) EBOLA GP Nsp
PERCENT SURVIVING
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10
DAYS POST-CHALLENGE

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/00860

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/39 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 01162 A (THE JHONS HOPKINS UNIVERSITY) 15 January 1998 see page 20 - page 21 ---	1,13,18
Y	KIM J J ET AL: "In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen." JOURNAL OF IMMUNOLOGY, (1997 JAN 15) 158 (2) 816-26, XP002103878 see the whole document ---	1-27
Y	WO 97 32987 A (UNIVERSITY OF TORONTO) 12 September 1997 see the whole document --- -/--	1-27

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[illegible]ional Application No
PCT/US 99/00860

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROY K ET AL: "DNA-CHITOSAN NANOSPHERES: TRANSFECTION EFFICIENCY AND CELLULAR UPTAKE" PROCEEDINGS OF THE 24TH. INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE OF BIOACTIVE MATERIALS, STOCKHOLM, JUNE 15 - 19, 1997, no. SYMP. 24, 15 June 1997, page 673/674 XP002052412 CONTROLLED RELEASE SOCIETY see the whole document ---	1-27
P,X	WO 98 08947 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 5 March 1998 see the whole document -----	1,13,18

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/00860

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 13-17 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 99/00860

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9801162 A	15-01-1998	AU 3739697 A	02-02-1998
WO 9732987 A	12-09-1997	AU 1865297 A	22-09-1997
WO 9808947 A	05-03-1998	AU 5507098 A	19-03-1998